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PROTEOLYSIS AS A FUNCTION OF DISTANCE FROM SURFACE TO CENTRE IN A SMEAR-RIPENED IRISH FARMHOUSE CHEESE

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ABSTRACT

This study focused on proteolysis in an Irish farmhouse smear-ripened cheese by serial slicing (0.41 mm/slice) the first 2 cm from surface towards the centre of the cheese. Urea-polyacrylamide gel electrophoretograms confirmed higher proteolysis in the outer layers than at the centre. Free amino acid (FAA) analysis confirmed decrease in proteolytic activity from surface to centre. Peptides produced at depths 0.41 mm and 20.5 mm were 720 and 427 from α_1 -casein; 691 and 337 from α_2 -casein; 807 and 453 from β -casein; 180 and 109 from κ -casein. The study confirms higher proteolytic activity at surface due to action of enzymes of the smear microbiota, than at the centre of cheese and identified the agents responsible for production of many peptides.

Keywords: Gubbeen, smear-ripened cheese, proteolysis, surface, mass spectrometry

1. INTRODUCTION

Smear-ripened, or bacterial surface ripened, cheeses are semi-soft varieties with an orange-red rind. As the name suggests, smear ripened cheeses (e.g., Gubbeen, Reblochon, Tilsit, brick and Taleggio) develop on their surfaces during ripening a viscous, microbial red-orange smear composed of bacteria and yeast (MOUNIER *et al.*, 2005; GOBETTI *et al.*, 1997). Towards the end of the ripening of the mature red-smear cheese surface, Gram-positive bacteria, yeasts (*Debaryomyces hansenii*) are predominant microorganisms present (BOCKELMANN *et al.*, 2002; BRENNAN *et al.*, 2004; RITSCHARD *et al.*, 2018). This microbial complexity is mainly responsible for the development of the characteristic flavours in the cheeses (CORSETTI, *et al.*, 2001; VALDÉS-STAUBER and SCHERER, 1997). During the first days of ripening of smear-ripened cheeses acid-tolerant yeasts grow and metabolize lactic acid produced by the lactic starter cultures to CO₂ and H₂O, thereby increasing the pH and producing growth factors, such as pantothenic acid, that encourages the growth of the smear bacteria (ELISKASES-LECHNER and GINZINGER 1995; PRILLINGER *et al.*, 1999; VALDÉS-STAUBER *et al.*, 1997; WYDER and PUHAN, 1999). *Debaryomyces hansenii* is the dominant yeast found in Tilsit cheese (CHURCHILL *et al.*, 2003; COGAN *et al.*, 2014). The yeasts isolated from the surface of Limburger cheese grew between pH 3.3 and 8.5 (KELLY and MARQUARDT, 1939; REPS, 1993) and *Mycoderma* yeasts isolated from the surface of Brick cheeses grow from pH 3 to 8 (IYA and FRAZIER, 1949; REPS, 1993). Presence of smear organisms on the surface of smear-ripened cheeses is associated with development of its colour, flavour and characteristic aroma (ADES and CONE 1969; RATTRAY and FOX, 1999).

Although *Brevibacterium linens* was considered in the past as the dominant bacterium on red-smear cheese, recent studies have shown a number of other species, particularly of genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Microbacterium* and *Rhodococcus* (REPS, 1993; ELISKASES-LECHNER and GINZINGER 1995; SEILER, 1986; VALDES-STAUBER *et al.*, 1997) are more important at the surface. Several bacterial species more recently isolated during the ripening of the smear cheeses include *Argococcus casei*, *Arthrobacter arilaitensis*, *Arthrobacter bergerei*, *Brachybacterium alimentarium*, *Brachybacterium tyroformantans*, *Corynebacterium casei*, *Microbacterium gubbeenese*, *Mycetocola reblochoni*, *Staphylococcus succinus* subsp. *casei* and *Staphylococcus equorum* subsp. *linens* (BORA *et al.*, 2007; BORA *et al.*, 2008; BRENNAN *et al.*, 2001; IRLINGER *et al.*, 2005; PLACE *et al.*, 2003; SCHUBERT *et al.*, 1996).

Species of bacteria occasionally isolated from smear cheese include *Staphylococcus* spp., *Brevibacterium aurantiacum* in very high numbers, and relatively high numbers of *Psychrobacter* spp. *C. casei*, *C. variabile*, and *M. gubbeennse*. The presence of *S. saprophyticus* in certain varieties (e.g., Gubbeen) distinguishes them from other varieties (Livarot, Reblochon, and Limburger) (COGAN *et al.*, 2014; REA *et al.*, 2007).

Proteolysis is the key biochemical process in cheese during ripening (FOX, 1993). The complex proteolytic breakdown from surface towards the centre of smear-ripened cheese is a result of the combined action of bacterial and yeast proteolytic enzymes. Proteolysis in this type of cheese depends directly on the extent of penetration of the smear microbiota in the cheese curd and their proteolytic activity and decreases inwards from the surface towards the centre (CHURCHILL *et al.*, 2003). Considerable differences in degree of proteolysis are observed, depending on depth from surface to centre of the cheese (CHURCHILL *et al.*, 2003). Smear cheese microbiota (bacteria, moulds and yeasts) and their enzyme systems plays a key role in proteolytic, lipolytic and free amino acid catabolic activities in the cheese during ripening (FOX, 1993). Yeasts present in the surface

smear, *D. hansenii* and *G. candidum*, exhibit a strong extracellular proteolytic and or peptidolytic activity (BAUR *et al.*, 2015). An *in situ* study by BOUTROU *et al.* (2006) found *G. candidum* from the surface of the cheese participates in primary proteolysis along with chymosin at pH 5.5 to 6 (CHEN and LEDFORD, 1972; GUEUEN and LENOIR, 1976) during the proteolysis. Rapid hydrolysis of α_s -caseins at the surface, in presence of *G. candidum* and surface microbiota of the cheese was observed also by BOUTROU *et al.* (2006).

Proteolytic enzymes of one of these organisms, *B. linens*, were studied by RATTRAY and FOX (1998). The purified proteinase enzyme from *B. linens* was most active at pH 8.5 and at 50°C with a molecular mass 120 kDa and peptides produced by these proteinases from α_s - and β - caseins were identified (RATTRAY and FOX, 1996, 1997, 1998).

However, there is relatively little literature describing in detail proteolysis and breakdown of caseins in smear-ripened cheese. The current study attempts to characterise proteolysis in a smear ripened variety of Irish farm-house cheese Gubbeen from the surface towards the centre. The study gives an insight to the detail of the proteolysis in smear cheese providing a deeper and more comprehensive insight into the metabolic activity of microbial ecosystems of these varieties.

2. MATERIALS AND METHODS

Commercially ripe samples (300 g) of Gubbeen, an Irish farmhouse smear cheese, product of Gubbeen Farmhouse Products Ltd., Gubbeen House, Schull, Co. Cork. Ireland), were obtained in triplicate from the local market (G1, G2 and G3), made generally as outlined by MOUNIER *et al.* (2005). These were cut in half along the diameter of the cheese wheel. One portion was crumbled and mixed thoroughly (with rind) for compositional analysis and remaining part was used for serial slicing and proteolytic analysis (Fig. 1 A, B, C and D).

2.1. Serial slicing of cheese and peptide extraction

The cheese. Four or five blocks from each cheese were sliced separately and slices taken at the same depth were later pooled. A tissue chopper (McIlwain tissue chopper; Model TC752, Campden Instruments Ltd., Loughborough, Leics, England) with a double end razor blade (Wilkinsons Sword, Edgewell Personal Care, Shelton, CT, USA) was set to give a thickness of 410 mm (0.410 mm) per slice. The blade was cleaned with 70% ethanol before and after each sectioning. Cheese blocks were sliced frozen, at thickness of 0.41 mm/slice, to a depth of 2 cm (Fig 1 A, B, C and D). These slices were collected on aluminium foil sheet with a sterile scalpel and an artist's brush. Slices from multiple blocks from a single cheese were pooled to get a sufficient quantity (10 mg) for analysis and stored frozen in a micro-centrifuge tube. The pooled slices were weighed out (10 mg), then dispersed in 10 mM citrate buffer (pH 5.5), teased using a needle and vortexed (RotaMixer, model # 8768; Hook and Tucker Instruments Ltd., Croydon, England) for 1 min (repeated if required) for uniform mixing. The micro-centrifuge tubes were held in a pre-heated water bath at 40°C for 15 min and centrifuged (Micro-Centaur Centrifuge, MB010.CX1.5, Sanyo, Loughborough, Leics, UK) at 9,600 g for 10 min, cooled for 30 min at 4°C to remove any fat from the supernatant. The supernatant was separated and sufficient 10 % trichloroacetic acid (TCA) solution was added to the supernatant to give final concentration of 2 % TCA in the extracts, which were centrifuged again at 6,700 g for 10

min and cooled at 4°C for 15 min. The clear supernatant was separated and stored frozen in aliquots at -20°C until use. The aliquots were thawed and filtered through 0.22 µm cellulose acetate filters (Sartorius GmbH, Gottingen, Germany) for peptide analysis by mass spectrometry (Q-ToF LCMS) as described below.

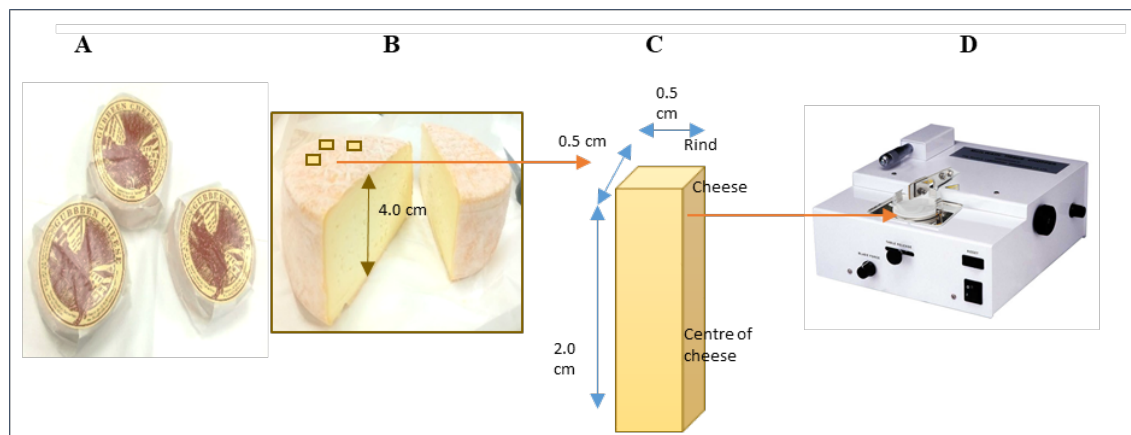


Figure 1. Sampling of smear ripened cheese to depth of 2 cm.

A: Sampling procedure for smear-ripened cheese; B: Cheese cut in halves; C: Blocks cut for slicing; D: McIlwain tissue chopper and slicing mantle.

2.2. Physico-chemical analysis

Compositional analysis of the cheeses comprised of determination of moisture content using an oven-drying method (IDF, 1982). Protein and nitrogen content of the cheeses and pH 4.6-soluble extracts were determined by the macro-Kjeldahl method ($N \times 6.38$; IDF, 1986). Percentage of fat was determined by the Gerber method (IIRS, 1955) and percentage of NaCl was measured by a titrimetric method using potentiometric end-point determination as described by FOX (1963). Each experimental analyses were run in triplicate for each cheese sample. Statistical analyses were performed using R® 16 (R version 3.4.0; the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand). Differences in means in between the batches and/or depth were tested by analysis of variance (one way-ANOVA) at significance level, α , of 0.05 (P value ≤ 0.05), throughout the study.

2.3. Proteolytic analysis

The pH of a cheese slurry (made from 25 g cheese and 50 g of deionised water) was measured using a calibrated pH meter. The same slurry was used to prepare pH 4.6-soluble and -insoluble fractions of cheeses at all time points as described by KUCHROO and FOX (1982).

Proteolysis in the slices taken from surface to centre of smear-ripened cheeses was studied by urea-polyacrylamide electrophoresis (urea-PAGE) of freeze-dried samples (10 mg/mL) of cheese slices from surface to centre (ANDREWS, 1983; SHALABI and FOX 1987; O'MAHONY *et al.*, 2005). The gels were stained with Coomassie Brilliant Blue G250 (BLAKESLEY and BOEZI, 1977) and de-stained by several distilled water washes.

Individual free amino acids (FAAs) content was determined according to FENELON *et al.* (2000). Frozen extracts of cheese slices taken at depths 0.41 mm, 1.64 mm and 20.5 mm were dispersed in 10 mM citrate buffer, pH 5.5, in 2% TCA, and were used for the analysis of all three batches.

Peptide analysis from cheese extracts (Section 2.1) were conducted at Conway Institute of Biomedical and Biomolecular Sciences, University College Dublin, Ireland, on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific, Waltham, MA, USA) mass spectrometer equipped with a reversed-phase Nano LC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific Waltham, MA, USA). Peptide samples were loaded onto C18 reversed phase columns (10 cm length, 75 μ m inner diameter) and eluted with a linear gradient from 2 to 97% acetonitrile containing 0.5% acetic acid in 60 min at a flow rate of 250 nL/min. The injection volume was 5 μ l. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2 acquisition. Survey full scan MS spectra (m/z 300-2000) were acquired in the Orbitrap. The twelve most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation.

2.4. Peptide identification

Raw data from the mass spectroscopy (Orbitrap Q-Exactive) were processed using MaxQuant version 1.5.5.1 (COX and MANN, 2008; TYANOVA *et al.*, 2016a), incorporating the Andromeda search engine (COX *et al.*, 2011). MS/MS spectra were matched to a bovine proteins custom database of previously identified entries (1,059) in the milk proteome in order to identify peptides and proteins. All searches were performed with unspecific digest. The database searches were performed with no fixed modification but with acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant, namely a false discovery rate of 1% at the peptide level. For the generation of ion intensities for peptide profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min (COX *et al.*, 2014). The Perseus statistical software (version 1.5.5.3) (TYANOVA *et al.*, 2016) was used to analysis the peptide ion current intensities.

3. RESULTS

3.1. Physico-chemical composition

The physico-chemical composition of three batches (G1, G2 and G3) of commercial smear-ripened cheese was determined (Table 1). pH decreased from 6.3 to 5.7 from surface to centre of the cheese, respectively, in all the batches, as was also reported by MOUNIER *et al.* (2005). Levels of moisture, fat, salt (NaCl), fat in dry matter (FDM), moisture in non-fat solids (MNFS), protein and nitrogen content in cheese and pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen (TN), are reported in Table 1. Moisture levels were 44 to 46% in agreement with the literature (MOUNIER *et al.*, 2005; REA *et al.*, 2007; COGAN *et al.*, 2014). Salt (% NaCl) was ~2.1 %, with no significant variation between batches. The % fat values were similar in all the batches (47 %) as were the values of % FDM (84% to 86%), %MNFS (83% to 87%). The gross composition was observed to be typical of smear ripened

cheese (COGAN *et al.*, 2014). Crude protein (N x 6.38) content of cheese showed a slight variation between the batches G1, G2 and G3 (Table 1).

Table 1. Physico-chemical compositional analysis of mature smear-ripened cheese after ripening.

	% Moisture	%Fat	%Salt	%N	%(Prt)	%pH4.6SN/TN	%MNFS	%FDM
G1	44.11±0.5 ^a	47±0.01 ^a	2.13±0.05 ^b	2.93±0.05 ^a	19.67±0.34 ^a	9.73±0.11 ^b	83.21±0.95 ^a	84.09±0.76 ^a
G2	44.31±1.4 ^a	47±0.02 ^a	2.03±0.05 ^a	3.36±0.15 ^b	21.41±1.00 ^b	6.56±0.66 ^a	83.61±2.81 ^a	84.43±2.22 ^a
G3	46.27±0.7 ^a	47±0.01 ^a	2.07±0.05 ^a	3.59±0.07 ^b	22.07±0.48 ^b	6.41±0.17 ^a	87.33±1.32 ^a	86.85±1.15 ^a

Samples G1, G2 and G3 are separate batches of smear-ripened cheese used for compositional analysis, where %N= Nitrogen%, %(Prt) = crude protein% in smear-ripened cheese, total SN= pH 4.6-soluble nitrogen (SN) as a percentage of total nitrogen, %FDM= fat in dry matter and % MNFS=moisture in non-fat solids, represented by mean ± standard deviation and different superscript letters represent significant difference (P<0.05).

3.2. Proteolysis

The ratio between nitrogen soluble at pH 4.60 and total nitrogen (pH 4.6 SN/TN) (%) is an index of proteolysis and, in smear-ripened cheese, is mainly produced by the action of chymosin, plasmin, lactocepins (LacCeps) and enzymes from the smear microbiota on the caseins (ARDO *et al.*, 2017). Levels of pH 4.6 SN/TN were higher in batch G1 as compared to batches G2 and G3 (Table 1). Overall % pH 4.6 SN/TN indicated a normal proteolysis at the end of the ripening in the cheese and values from the current study were comparable to those reported by COGAN *et al.* (2014) a range of smear-ripened cheeses.

Proteolysis from surface to the centre of the cheese was also studied by urea-PAGE (Fig. 2). Samples for the gel were prepared from freeze dried slices (0.41 mm/ slice) of smear-ripened cheese from surface to centre (2 cm). Extensive breakdown of α_{s1} - and β -casein was observed in topmost layers up to 1.23 mm, followed by normal patterns of proteolysis typical of internal bacterially ripened varieties, closer to the centre. Action of plasmin was prominent for the break-down of β -caseins into γ -caseins at depth less than 1 cm from the outer surface, as was also observed in other smear ripened cheese varieties like Tilsit at early stages (day 6) of ripening (BOUTROU *et al.*, 1999, 2005, 2006; GUIZANI *et al.*, 2002; CHURCHILL *et al.*, 2003). Extensive degradation of α_{s1} -casein at the surface (Fig. 2) was likely caused by the action of chymosin together with the enzymes from the smear microbiota. Levels of degradation decreased from surface to center of the cheese, due to reduced penetration of smear enzymes. The high extent of degradation of α_{s1} -casein at the surface despite the higher pH (which would reduce chymosin action) suggests a major role of enzymes from smear organisms. The results are similar to those observed by CHURCHILL *et al.* (2003) in Tilsit cheese.

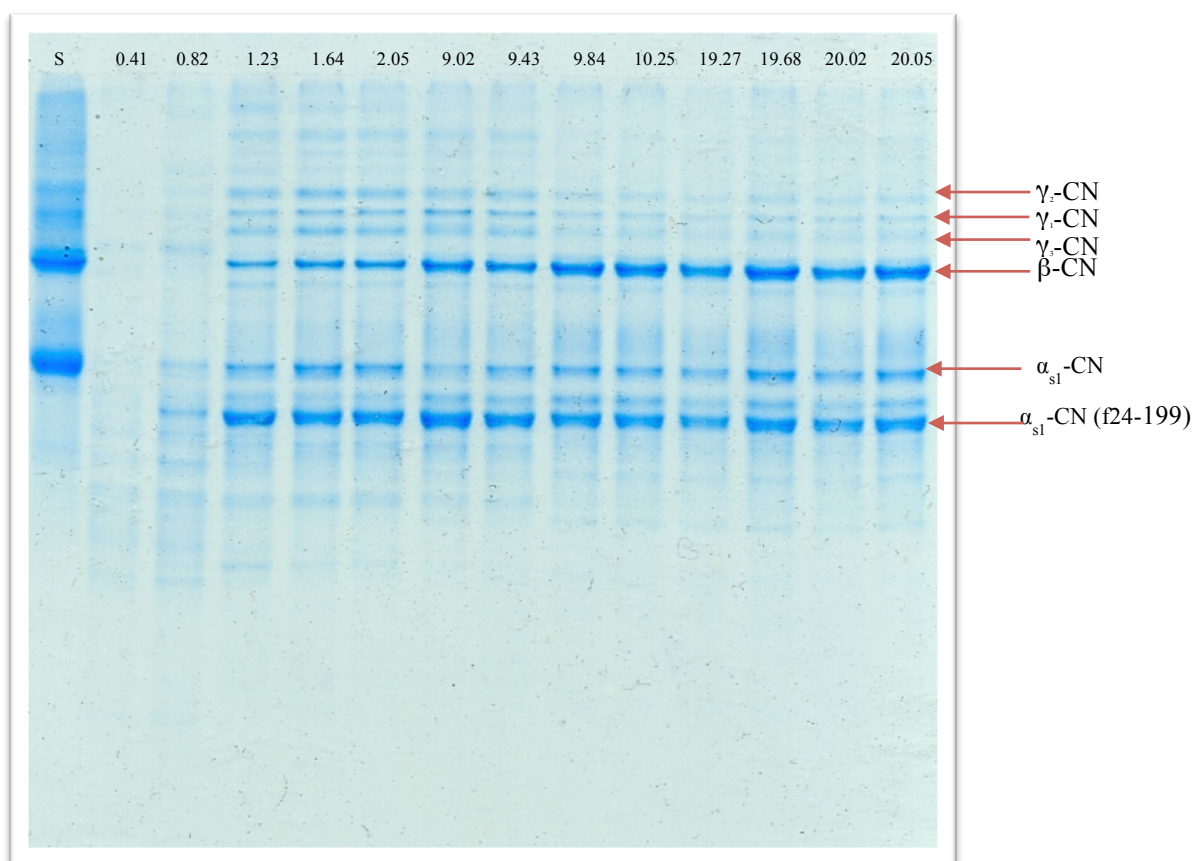


Figure 2. Urea-polyacrylamide gel electrophoretograms of freeze dried samples of sliced smear-ripened cheese from surface to centre (2 cm), standard protein marker, sodium caseinate: (S), numbers above lanes 2-14 indicates depth of smear-ripened cheese in mm at which sample was taken.

Profiles of free amino acids analysed from extracts from cheeses dispersed in 10 mM citrate buffer at pH 5.5 are as shown in Fig. 3. Total amino acid contents observed were 107.19 $\mu\text{g}/\text{mg}$ at 0.41 mm, 33.59 $\mu\text{g}/\text{mg}$ at 1.64 mm and 33.78 $\mu\text{g}/\text{mg}$ at 20.5 mm depth of cheese. Levels of individual amino acids were also found to be higher at the surface than towards centre (Fig. 3). Values at 0.41 mm for glutamic acid were 21.60 $\mu\text{g}/\text{mg}$, glycine were 16.5 $\mu\text{g}/\text{mg}$, proline were 16.56 $\mu\text{g}/\text{mg}$ and of alanine were 10.03 $\mu\text{g}/\text{mg}$ (Fig. 2). The outer layers of the cheese showed high levels of glutamic acid, glycine, alanine, tyrosine, and proline only particular to the surface of the smear cheese. Higher concentrations of threonine, lysine, phenylalanine and valine were found towards the inner layers of smear-ripened cheese than at the surface. Values were similar also to amino acid profiles of mould ripened cheeses (ZARMPOUTIS *et al.*, 1997).

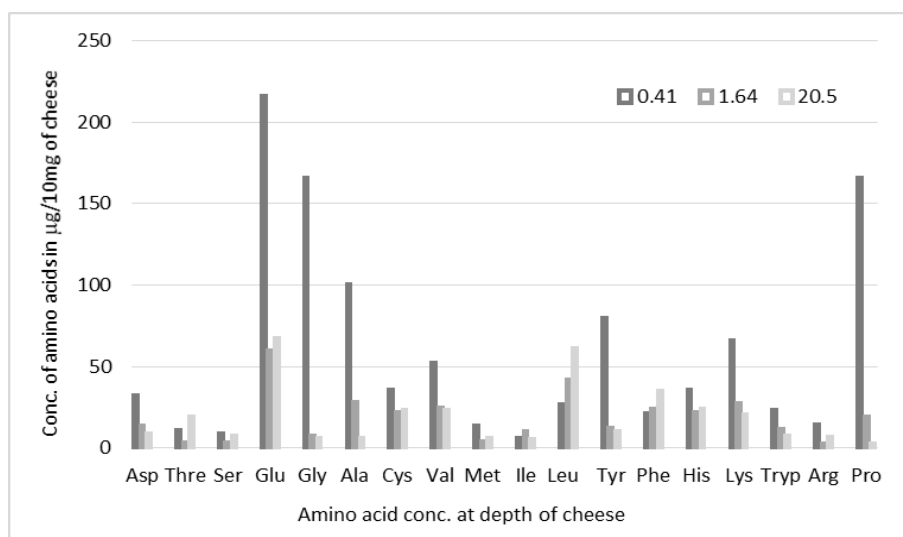


Figure 3. Free amino acid analysis.

Concentration ($\mu\text{g} / \text{mg}$) of free amino acids in 10 mM citrate buffer pH 5.5 dispersed extracts from 0.41 mm, 1.64 mm and 20.5 mm slices of smear-ripened cheese.

3.3. Peptide profiles at different depths of the cheese

Qualitative differences were found in peptides produced at all depths. Mass spectroscopic analysis of peptides in 10 mM citrate extracts at pH 5.5 showed a large difference between the numbers of peptides produced at the outer surface as compared to the centre (depth of ~2 cm) (Fig. 4). In case of the individual caseins, the number of β -casein-derived peptides was highest at all the depths, followed by peptides derived from α_{s1} -casein, α_{s2} - and κ -casein.

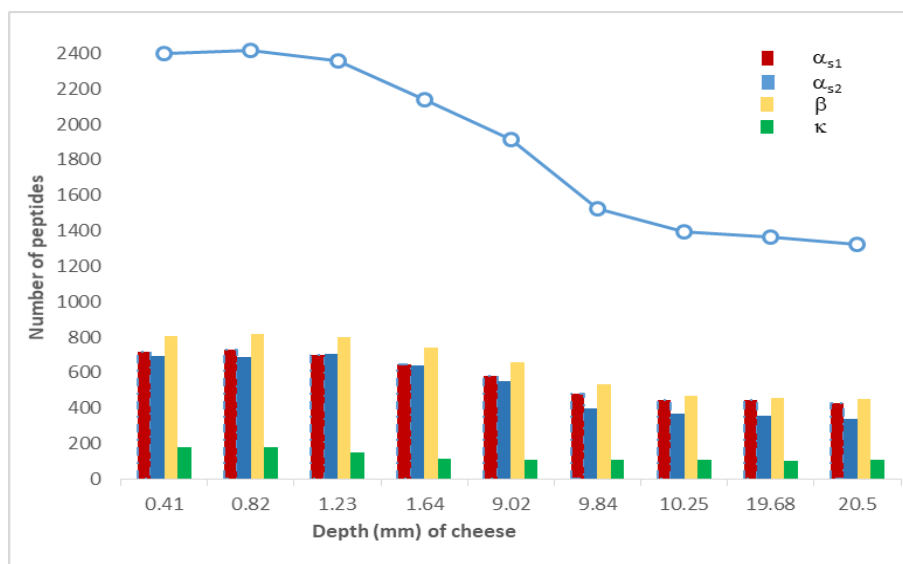


Figure 4. Total caseins at depths in smear ripened cheese.

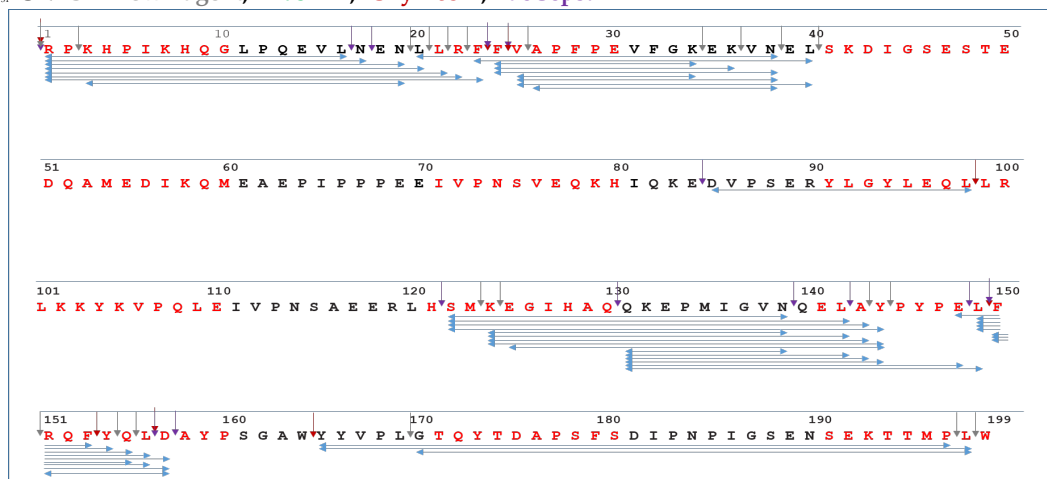
Graph representing the number of casein derived-peptides as a function of distance from surface of cheese (-O-). Bars indicate number of peptides derived from α_{s1} -, α_{s2} -, β - and κ -casein.

Identification of peptides during ripening was a major objective of this study. So far, very little has been reported in the literature about identification of peptides from smear cheese. The first 150 peptides with highest values of relative intensity at each depth, obtained from LCMS data were considered and sorted into peptides from α_1 -casein, α_2 -casein, β -casein and κ -casein at depths 0.41 mm, 9.02 mm and 20.05 mm and were plotted on the primary structure of the proteins (Fig. 5 A, B, C and D). Identified peptides were compared with known cleavage specificities of chymosin, plasmin and lactocepins (SINGH *et al.*, 1994, 1995, 1997; BREEN *et al.*, 1995; FERNANDEZ *et al.*, 2005; UPADHYAY *et al.*, 2006; ARDO *et al.*, 2017). The peptides were also compared with the cleavage specificities of proteinases of *B. linens* on α_1 - and β -casein found by RATTRAY and FOX (1993). However, cleavage site matches were not found in first 150 peptides from the extracts of smear cheese.

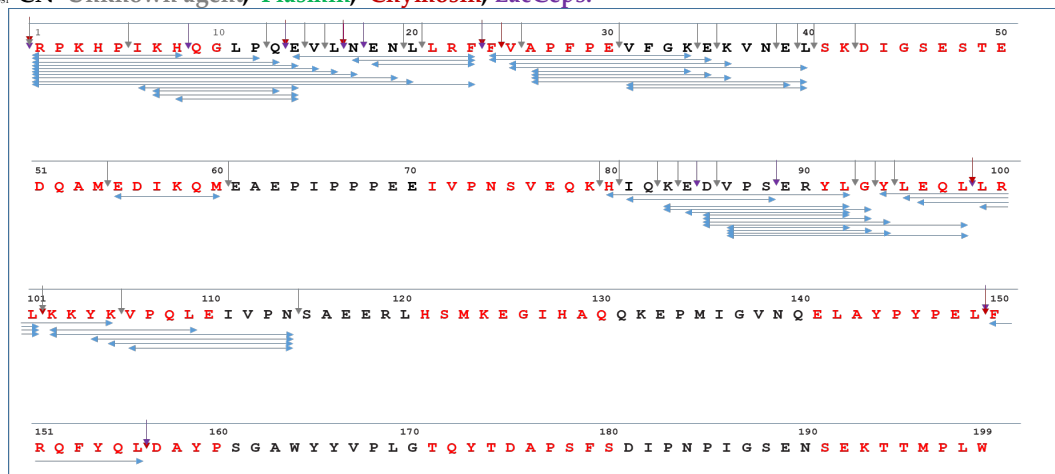
A total of 5176 α_1 -casein fragments were produced by hydrolysis during ripening. Peptides derived from α_1 -casein only at 0.41 mm were observed in regions 121-157 and 165-198. Those derived only at 9.02 mm and 2.05 mm and were found at regions 79-117. The region hydrolysed common to all depths, (0.41 mm, 9.02 mm and 2.05 mm) was 1-40 (Fig. 5 A). All these regions were highly susceptible to enzymatic hydrolysis for action of chymosin and lactocepins (ARDO *et al.*, 2017) and proteinases from surface smear microbiota. The common fragment, α_1 -CN (f1-23) produced by the action of chymosin (FOX and McSWEENEY, 1996), was present at all depths. Other major chymosin-derived peptides, α_1 -CN (14-23), α_1 -CN (f25-36/38), α_1 -CN (f17-23/24), α_1 -CN (f 24-34) and α_1 -CN (f 25-34), were found at all depths. Peptides produced by action of lactocepins and/or chymosin at their N- or C- termini (ARDO *et al.*, 2017) included α_1 -CN (f30-38), α_1 -CN (f31-37/38), α_1 -CN (f33-40), α_1 -CN (f154-164), α_1 -CN (f165-193), α_1 -CN (f169-189/190/197/198), α_1 -CN (f170-189/190/192/198) and α_1 -CN (f180-198/199). Lactocypin-derived peptide fragments found at all the depths were α_1 -CN (1-8/9/13/16/17), α_1 -CN (f7-13), α_1 -CN (f10-16), α_1 -CN (f122-139,142/143/144) and α_1 -CN (131-139/142/148). Most common peptides from α_1 -casein present only at surface were α_1 -CN (f 122-139/142/143), α_1 -CN (f 124-142/143/144), α_1 -CN (f 125-144) and α_1 -CN (f 150-155/156/157) and those present only at the center were α_1 -CN (f191/193-199). Peptides from α_1 -casein present at all the depths were α_1 -CN (f1-23), α_1 -CN (f14-23), α_1 -CN (f 24-34) and α_1 -CN (131-139/142/148).

A total of 4744 peptides were produced by hydrolysis of α_2 -casein during ripening. Peptides derived from α_2 -casein only at depth 0.410 mm were from region 68-98, those common to depths 0.410 mm and 9.02 mm were from the regions 98-114 and 175-207 and peptides common at depths 9.02 mm and 2.05 mm were found in regions 89-99, 102-115 and 181-207 (Fig. 5 B). Peptides produced at all the depths were mainly due to the action of plasmin, lactocepins (ARDO *et al.*, 2017), chymosin (McSWEENEY *et al.*, 1994) and mainly at the surface by proteinases from the smear microbiota. Fragments produced by the action of plasmin and lactocepins at their N-termini were α_2 -CN (f115-125) and α_2 -CN (f115-126). Action of plasmin and lactocepins (ARDO *et al.*, 2017) on α_2 -casein helped to produce fragments α_2 -CN (f80-88), α_2 -CN (f150-161/162), α_2 -CN (f151-156/158/161/162/163) and α_2 -CN (f196-208), which were also found in smear ripened cheeses at all depths. Most common peptides from α_2 -casein present only at surface were α_2 -CN (f68-74/88/94), α_2 -CN (f71-94/98), α_2 -CN (f73-86/88), α_2 -CN (f75-88/94) and α_2 -CN (f87-95) and those present only at the center were, α_2 -CN (f176-181/182), α_2 -CN (f186-197), α_2 -CN (f188-199/202), α_2 -CN (f191-197/199/203) and α_2 -CN (f194-203). Peptides from α_2 -casein present at all the depths were α_2 -CN (f115-125), α_2 -CN (f151-156/158/161/162/163) and α_2 -CN (f196-208).

0.41mm α_{s1} -CN Unknown agent; Plasmin; Chymosin; LacCeps.



0.41mm α_{s1} -CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm α_{s1} -CN Unknown agent; Plasmin; Chymosin; LacCeps.

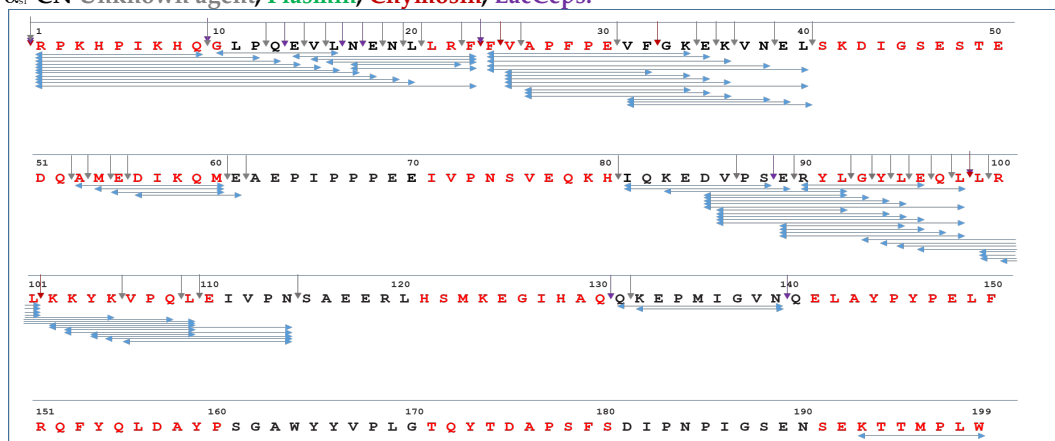


Figure 5 A. The primary structure of bovine α_{s1} -casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

EN Unknown agent, **Fluorin**, **Chrysofin**, **Laccase**.

1 10 20 30 40 50
K N T M E H V S S S E E S I I S Q E T Y K Q E K N M A I N P S K E N L C S T F C K E V V R N A N E E

51 60 70 80 90 100
E Y S I G S S S E E S A E V A T E E V K I T V D D K H Y Q K A L N E I N E F Y Q K F P Q Y L Q Y L Y

101 110 120 130 140 150
Q G P I V L N P W D Q V K R N A V P I T P T L N R E Q L S T S E E N S K K T V D M E S T E V F T K K

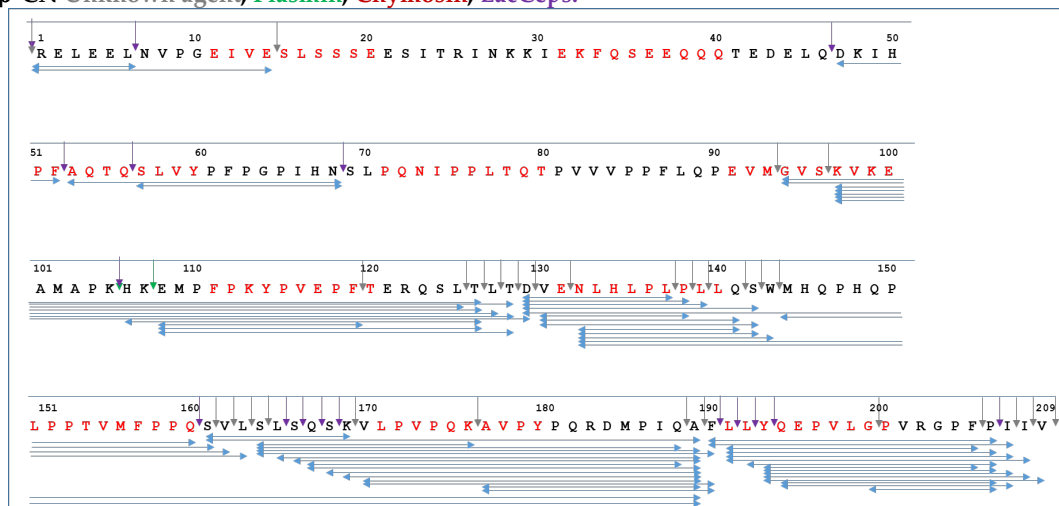
151 160 170 180 190 200 207
T K L T E E E K N R L N F L K K I S Q R Y Q K F A L P Q Y L K T V Y Q H Q K A M K P W I Q P K T K V I P Y V R Y L

1 10 20 30 40 50
 K N T M E H V S S S E E S I I S Q E T Y K Q E K N M A I N P S K E N L C S T F C K E V V R N A N E E
 51 60 70 80 90 100
 E Y S I G S S S E E S A E V A T E E V K I T V D D K H Y Q K A L N E I N E F Y Q K F P Q Y L Q Y L Y
 101 110 120 130 140 150
 Q G P I V L N P W D Q V K R N A V P I T P T L N R E Q L S T S E E N S K K T V D M E S T E V F T K K
 151 160 170 180 190 200 207
 T K L T E E E K N R L N F L K K I S Q R Y Q K F A L P Q Y L K T V Y Q H Q K A M K P W I Q P K T K V I P Y V R Y L

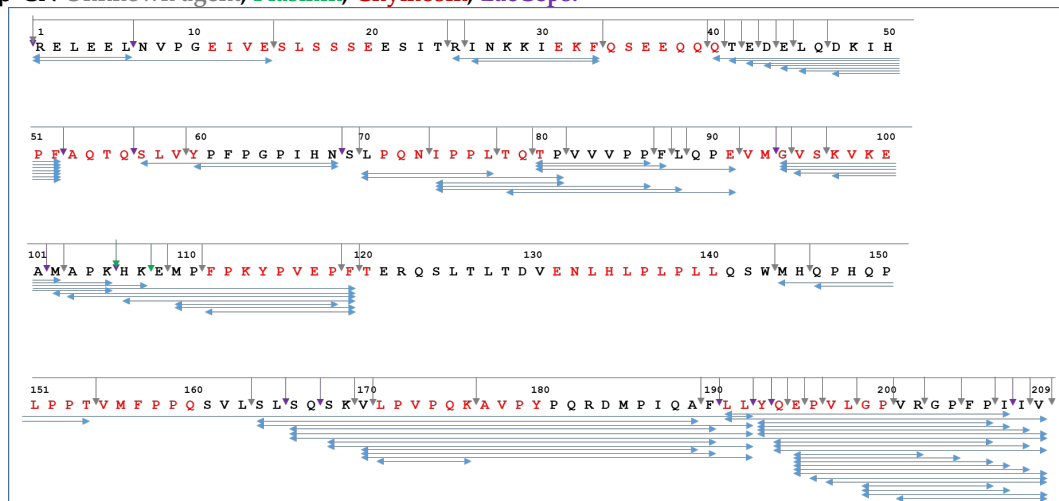
1 10 20 30 40 50
 K N T M E H V S S S E E S I I S Q E T Y K Q E K N M A I N P S K E N L C S T F C K E V V R N A N E E
 51 60 70 80 90 100
 E Y S I G S S S E E S A E V A T E E V K I T V D D K H Y Q K A L N E I N E F Y Q K F P Q Y L Q Y L Y
 101 110 120 130 140 150
 Q G P I V L N P W D Q V K R N A V P I T P T L N R E Q L S T S E E N S K K T V D M E S T E V F T K K
 151 160 170 180 190 200 210
 T K L T E E K N R L N F L K K I S Q R Y Q K T A L P Q Y L K T V Y Q H Q A M K P W I Q P K T K V I P Y V R Y L

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0.41 mm β -CN Unknown agent; Plasmin; Chymosin; LacCeps.



9.02 mm β -CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm β -CN Cheese: Unknown agent; Plasmin; Chymosin; LacCeps.

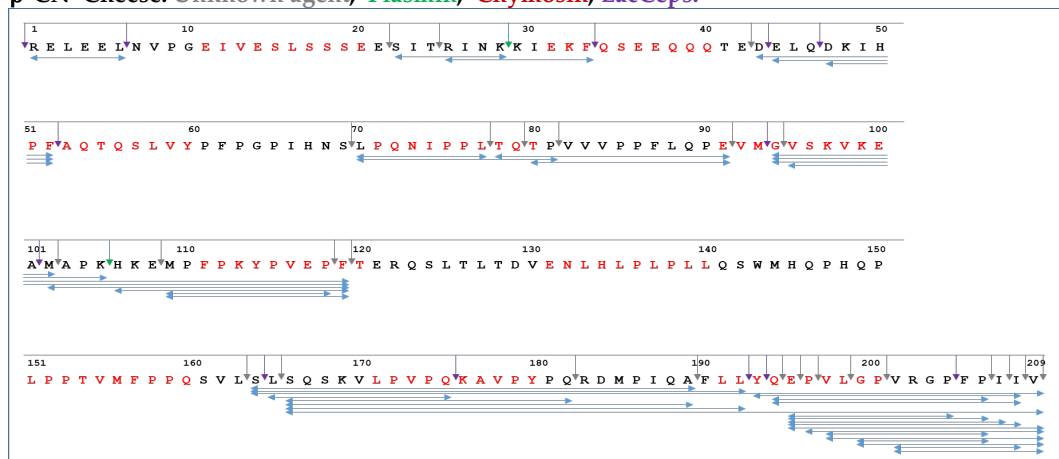
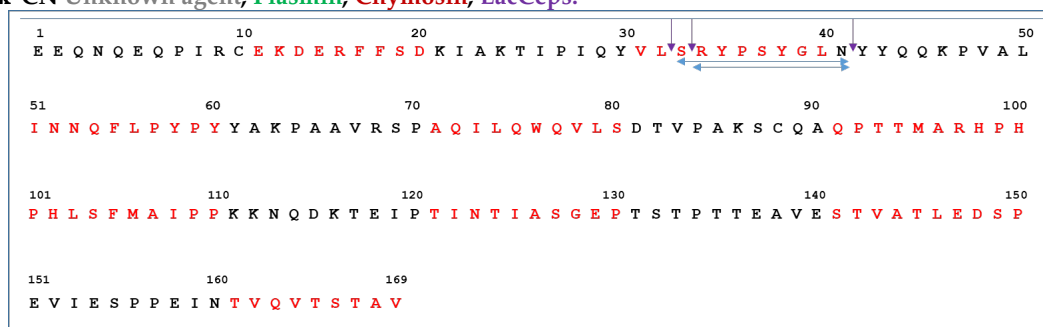


Figure 5 C. The primary structure of bovine β -casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactocepins (LacCeps).

0.41 mm κ -CN Unknown agent; Plasmin; Chymosin; LacCeps.



9.02 mm κ -CN Unknown agent; Plasmin; Chymosin; LacCeps.



20.05 mm κ -CN Cheese: Unknown agent; Plasmin; Chymosin; LacCeps.

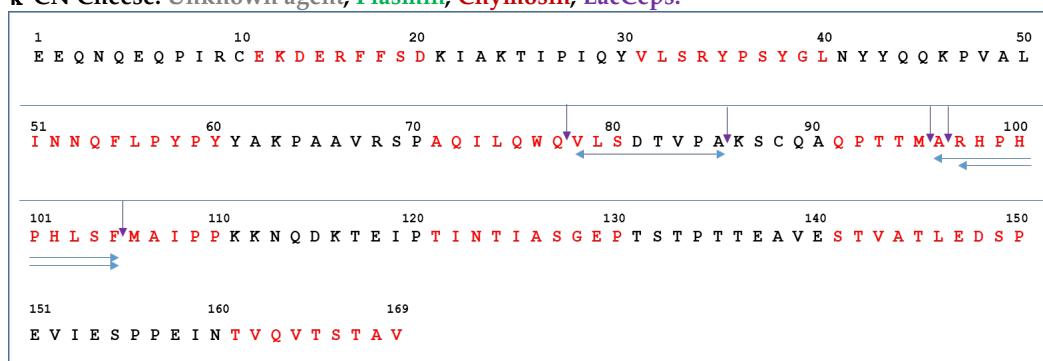


Figure 5 D. The primary structure of bovine κ -casein, showing the peptides in 10 mM citrate buffer pH 5.5-dispersed extracts of Gubbeen cheese slices taken at depth 0.41mm, 9.02 mm and 20.05 mm, together with known cleavage sites of by action of unknown agent, plasmin (Pla), chymosin (Chy), or Lactoceps (LacCeps).

A total of 5740 peptides were produced by hydrolysis of β -casein during ripening (Supplementary Data Table 4). Peptides derived from β -casein found only at depth 0.410 mm were produced from regions 130-161 and those produced only at depth 9.02 mm and 20.05 mm were from regions 39-52 and 69-119. Peptides commonly produced at all depths were found at region 1-6, 164-190 and 193-207 (Fig. 5 C). Peptide fragments derived from action of only plasmin on N- and -C termini were β -CN (f22-28), β -CN (f94/97-105), β -CN (f106-119) and β -CN (f129-137) with action of proteinases from the smear microbiota. Similarly, peptide fragments produced by the action of only lactoceps were β -CN (f1-

6/14), β -CN (f45/47-52), β -CN (f53/57-68), β -CN (f132-160), β -CN (f164/170/177/190), β -CN (f165-175), β -CN (f166/176-182), β -CN (f166/168/192), β -CN (f191/193/194-207) and β -CN (f166/191/192-209). Most common peptides from b-casein present only at surface were β -CN (f 1-14), β -CN (f 53/57-68), β -CN (f 94-124/128), β -CN (f 95-125/126/127/128/129), β -CN (f 129/130/132-137/139/142), β -CN (f 132/142-161) and β -CN (f 144-162/163/189) and those present only at the center were, β -CN (f 22/23-28), β -CN (f 25-33), β -CN (f 70-77), β -CN (f 74-86), β -CN (f 78/80/84-91), β -CN (f 94-102/105), β -CN (f 95/95-119) and β -CN (f 201-204). Peptides from α_s -casein present at all the depths were β -CN (f22-28), β -CN (f166/176-182), β -CN (f166/168/192) and β -CN (f166/191/192-209).

A small number of peptides from κ -casein was found at low concentrations during ripening (Fig. 5 D). Fragments produced from pH 4.6-soluble extract, by action of lactocepins at their N- or C- termini (ARDO *et al.*, 2017) were κ -CN (f33-41), κ -CN (f78-85) and κ -CN (f96-103/105). κ -CN (f96-105) was produced by chymosin cleaving at its C-terminus (ARDO *et al.*, 2017). More detailed study on enzyme specificities of the smear ripened cheese can be a future area of research.

4. CONCLUSION

The gross composition of smear ripened cheese was observed to be typical of the cheese variety. Overall % pH 4.6 SN/TN in all three batches G1, G2 and G3, indicated a normal proteolysis at the end of the ripening in the cheese. Extent of proteolysis was observed by urea-PAGE which showed extensive breakdown of α_s - and β -casein essentially from surface to 1.23 mm, followed by normal patterns of proteolysis typical of bacterially ripened cheeses, towards the centre. Free amino acid analysis of surface layers of the cheese showed high levels of glutamic acid, glycine, alanine, tyrosine, and proline, whereas higher concentrations of threonine, lysine, phenylalanine and valine were found towards the inner layers of smear-ripened cheese. A total of 5614 peptides were identified during the study at depths 0.41mm, 9.02 mm and 20.05 mm.

Most common peptides from α_s -casein present only at surface were α_s -CN (f 122-139/142/143), α_s -CN (f 124-142/143/144), α_s -CN (f 125-144) and α_s -CN (f 150-155/156/157) and those present only at the center were α_s -CN (f191/193-199). Most common peptides from α_2 -casein present only at surface were α_2 -CN (f68-74/88/94), α_2 -CN (f71-94/98), α_2 -CN (f73-86/88), α_2 -CN (f75-88/94) and α_2 -CN (f87-95) and those present only at the center were, α_2 -CN (f176-181/182), α_2 -CN (f186-197), α_2 -CN (f188-199/202), α_2 -CN (f191-197/199/203) and α_2 -CN (f194-203). Most common peptides from b-casein present only at surface were β -CN (f 1-14), β -CN (f 53/57-68), β -CN (f 94-124/128), β -CN (f 95-125/126/127/128/129), β -CN (f 129/130/132-137/139/142), β -CN (f 132/142-161) and β -CN (f 144-162/163/189) and those present only at the center were, β -CN (f 22/23-28), β -CN (f 25-33), β -CN (f 70-77), β -CN (f 74-86), β -CN (f 78/80/84-91), β -CN (f 94-102/105), β -CN (f 95/95-119) and β -CN (f 201-204).

Common peptides from α_s -casein at all the depths were α_s -CN (f1-23), α_s -CN (14-23), α_s -CN (f 24-34) and α_s -CN (131-139/142/148); from α_2 -casein at all the depths were α_2 -CN (f115-125), α_2 -CN (f151-156/158/161/162/163) and α_2 -CN (f196-208); β -casein at all the depths were β -CN (f22-28), β -CN (f166/176-182), β -CN (f166/168/192) and β -CN

(f166/191/192-209) and those from κ -casein were κ -CN (f33-41), κ -CN (f78-85) and κ -CN (f96-103/105). κ -CN (f96-105).

Study of individual proteolytic enzymes and their effect on the caseins of smear ripened cheese and detailed study of microbiological aspects will be an interesting area for further research.

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REFERENCES

- Ades G.L. and Cone J.F. 1969. Proteolytic activity of *Brevibacterium linens* during ripening of Trappist type cheese. *Journal of Dairy Science* 52:957-961. DOI: doi.org/10.3168/jds.S0022-0302 (69)86674-9.
- Andrews A. T., 1983. Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research* 50:45-55. DOI: doi.org/10.1017/S0022029900032519.
- Ardö Y., Mcsweeney P.L.H., Magboul A.A.A., Upadhyay V.K. and Fox P.F. 2017. *Biochemistry of cheese ripening: Proteolysis. Cheese: Chemistry, Physics and Microbiology*. 4th Edn., P.L.M. McSweeney, P.F. Fox, P.D. Cotter and D.W. Everett, Elsevier Academic Press, London, UK; Vol. 1, 445-482.
- Baur C., Krewinkel M., Kranz B., et al., 2015. Quantification of the proteolytic and lipolytic activity of microorganisms isolated from raw milk. *International Dairy Journal* 49:23-29. DOI: doi.org/10.1016/j.idairyj.2015.04.005.
- Bikash C., Ghosh T., Sienkiewicz T. and Krenkel K. 2000. *Brevibacterium linens* - A useful enzyme producer for cheese: A review. *Milchwissenschaft*. 55:628-632.
- Blakesley R.W. and Boezi J.A. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry* 82:580-582. DOI: doi.org/10.1016/0:003-2697(77)90197-x.
- Bockelmann W. 2002. Development of defined surface starter cultures for the ripening of smear cheeses. *International Dairy Journal* 12:123-131. DOI: 10.1016/S0958-6946(01)00152-2.
- Bora N., Vancanneyt M., Gelsomino R., Swings J., Brennan N., Cogan T.M., Larpin S., Desmasures N., Lechner F.E., Kroppenstedt R.M., Ward A.C. and Goodfellow M., 2007. *Agrococcus casei* sp. nov., isolated from the surfaces of smear-ripened cheeses. *International Journal of Systematic and Evolutionary Microbiology* 57:92-97. DOI: doi.org/10.1099/ijs.0.64270-0.
- Bora N., Vancanneyt M., Gelsomino R., Snauwaert C., Swings J., Jones A.L., Ward A.C., Chamba J.-F., Kroppenstedt R.M., Schumann, P. and Goodfellow M., 2008. *Mycetocola reblochon* sp. nov., isolated from the surface microbial flora of Reblochon cheese. *International Journal of Systematic and Evolutionary Microbiology* 58:2687-2693. DOI: doi.org/10.1099/ijs.0.64201-0.
- Breen E.D., Fox P.F. and Mcsweeney P.L.H. 1995. Fractionation of peptides in a 10 kDa ultrafiltration retentate of a water-soluble extract of Cheddar cheese. *International Journal of Food Science* 7:211-220.
- Brennan N.M., Brown R., Goodfellow M., Ward A.C., Beresford T.P., Simpson P.J., Fox P.F. and Cogan T.M. 2001. *Corynebacterium mooreparkense* sp. nov., and *Corynebacterium casei* sp. nov. isolated from the surface of a smear-ripened cheese. *International Journal of Systematic and Evolutionary Microbiology* 51:843-852. DOI: doi.org/10.1099 /00207713-51-3-843.
- Brennan N.M., Brown R., Goodfellow M., Ward A.C., Beresford T.P., Vancanneyt M., Fox, P.F. and Cogan T.M. 2001. *Microbacterium gubbeenense* sp. nov., isolated from the surface of a smear-ripened cheese. *International Journal of Systematic and Evolutionary Microbiology* 51:1969-1976. DOI: doi.org/10.1099/00207713-51-6-1969.
- Boutrou R. and Gueguen M. 2005. Interests in *Geotrichum candidum* for cheese technology. *International Journal of Food Microbiology*, 102:1-20. DOI: doi.org/10.1016/j.idairyj.2005.07.007.

- Chen M.H. and Ledford R.A. 1972. Proteolytic activity of *Geotrichum candidum*. Journal of Dairy Science 55:666.
- Churchill M.M., Hannon J.A. and Mcsweeney P.L.H. 2003. Proteolysis at the Surface of Tilsit Cheese, *Milchwissenschaft*, 58(5-6):293-296.
- Cogan T.M., Jamet E., Beduhn R., Bora N., Chamba J.-F., Irlinger F., Sebastiani H., Desmasures N., Hohenegger M., Mounier J., Ward A.C., Goodfellow M., Goerges S., Guéguen M., Larpin S., Swings J., Vancanneyt M., Gelsomino R., Rea M.C. and Scherer S., 2014. Biodiversity of the surface microbial consortia from Limburger, Reblochon, Livarot, Tilsit, and Gubbeen cheeses. *Microbiology Spectrum* 2, CM-0010-2012. DOI: doi.org/10.1128/microbiolspec.CM-0010-2012.
- Corsetti A., Rossi J. and Gobbetti M. 2001. Interactions between yeasts and bacteria in the smear surface-ripened cheeses. *International Journal of Food Microbiology* 69:1-10. DOI: doi.org/10.1016/S0168-1605(01)00567-0
- Cox J. and Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* 26:1367-1372. DOI: doi.org/10.1038/nbt.1511.
- Cox J., Hein M. Y., Lubner C. A., Paron I., Nagaraj N. and Mann M. 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular and Cellular Proteomics* 13:2513-2526. DOI: doi.org/10.1074/mcp.M113.031591.
- Cox J., Neuhauser N., Michalsk A., Scheltema R. A, Olsen J. V. and Mann M. 2011. Andromeda: A peptide search engine integrated into the MaxQuant environment. *Journal of Proteome Research* 10(4):1794-805. DOI: doi.org/10.1021/pr101065j.
- Eliskases-Lechner F., and Ginzinger W. 1995. The yeast flora of surface ripened cheese. *Milchwissenschaft* 50:458-462.
- Eliskases-Lechner F., Kossler A., and Ginzinger W. 1997. The incidence and characterization of *Geotrichum* sp. in cheese. *Deutsche Molkerei-Zeitung, Lebensmittelindustrie und Milchwirtschaft* 118:56-61.
- Exterkate F. A. 1995. The lactococcal cell envelope proteinases: Differences, calcium-binding effects and role in cheese ripening. *International Dairy Journal* 5:995-1018. DOI: doi.org/10.1016/0958-6946(95)00042-9
- Exterkate F. A. 2000 Structural changes and interactions involved in the Ca²⁺- triggered stabilization of the cell-bound cell envelope proteinase in *Lactococcus lactis* subsp. *cremoris* SK11. *Applied and Environmental Microbiology* 66:2021-2028. DOI: doi.org/10.1128/AEM.66.5.2021-2028.2000
- Exterkate F. A., Slangen C. J., and Siezen R. J. 2001. Effect of genetically modified *Lactococcus lactis* cell-envelope proteinases with altered specificity on the course of casein degradation under cheese conditions. *International Dairy Journal* 11:363-371. DOI: doi.org/10.1016/S0958-6946(01)00065-6.
- Fenelon M. A. and Guinee T. P. 2000. Primary proteolysis and textural changes during ripening in Cheddar cheeses manufactured to different fat contents. *International Dairy Journal* 10:151-158.
- Gobbetti M., Lowney S., Smacchi E., Battistotti B., Damiani P. and Fox P.F. 1997. Microbiology and biochemistry of Taleggio cheese during ripening. *International Dairy Journal* 7:509-517. DOI: doi.org/10.1016/S0958-6946(97)00044-7.
- Guéguen M., and Lenoir J. 1976. Caractères du système protéolytique de *Geotrichum candidum*. *Lait* 557:439-448. DOI: doi.org/10.1051/lait:197655723.
- IDF 1982. Cheese and processed cheese; determination of the total solids content. Standard 4A:1982. Brussels, Belgium: International Dairy Federation.
- IDF 1986. Determination of the Nitrogen Content (Kjeldahl Method) and calculation of crude protein content. Standard 20A:1986. Brussels, Belgium: International Dairy Federation.
- IIRS 1955. Determination of the percentage of fat in cheese. Irish Standard 69:1955. Institute for Industrial Research and Standards, Dublin.
- Irlinger F. and Mounier J. 2009. Microbial interactions in cheese: implications for cheese quality and safety. *Current Opinions in Biotechnology* 20:142-148. DOI: 10.1016/j.copbio.2009.02.016.
- Iya K. K., and Frazier W. C. 1949. The yeast in the surface smear of Brick Cheese. *Journal of Dairy Science* 32:475.

- Kelleher P., Bottacini F., Mahony J., Kilcawley K. N., and van Sinderen D. 2017. Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. BMC genomics 18(1):267. DOI: doi.org/10.1186/s12864-017-3650-5
- Kelly C.D. 1939. The microbiological flora on the surface of Limburger cheese. Journal of Dairy Science 20:239-246. DOI: doi.org/10.3168/jds.S0022-0302 (37)95692-3.
- Knoop A. M. and Buchheim W. 1980. Different development of the structure in Harzer, Tilsit and Camembert -cheese during ripening. Milchwissenschaft, 35:482-488.
- Kuchroo C.N., and Fox P.F. 1982. Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. Milchwissenschaft, 37:331-335.
- Leclercq-Perlat M.-N., A. Oumer J.-L. Bergere H. E. Spinnler and Corrieu G.1999. Growth of *Debaryomyces hansenii* on a bacterial surface-ripened soft cheese. Journal of Dairy Research 66:271-281. DOI: doi.org/10.1017/S0022029999003362.
- Mounier J., Gelsomino R., Georges S., Vancanneyt M., Vandemeulebroecke K., Hoste B., Scherer S., Swings J., Fitzgerald G.F and Cogan T.M. 2005. Surface flora of four smear-ripened cheeses. Applied and Environmental Microbiology 71:6489-6500. DOI: doi.org/10.1128/AEM.71.11.6489-6500.2005.
- Place R. B., Hiestand D., Burri S. and Teuber M. 2002. *Staphylococcus succinus* subsp. *casei*, subsp. nov., a dominant isolate from surface-ripened semi-hard cheeses. Systematic and Applied Microbiology 25:353-359. DOI: doi.org/10.1078/0723-2020-00130.
- Place R. B., Hiestand D., Burri S. and Teuber M. 2003. *Staphylococcus succinus* subsp. *casei*, subsp. nov., a dominant isolate from *Staphylococcus equorum* subsp. *linens*, subsp. nov., a starter culture component for surface-ripened semi-hard cheeses. Systematic and Applied Microbiology 26:30-37. DOI: doi.org/10.1078/072320203322337281.
- Prillinger H., Molnar O., Eliskases-Lechner F. and Lopandic K. 1999. Phenotypic and genotypic identification of yeasts from cheese. Antonie van Leeuwenhoek. 75:267-283. DOI: doi.org/10.1023/A:100188991.
- Ratray F. P., Fox P. F. and Healy A. 1996. Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine α_s -casein. Applied and Environmental Microbiology 62:501-506.
- Ratray F. P., Fox P. F. and Healy A. 1997. Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine β -casein. Applied Journal of Environmental Microbiology 63:2468-2471.
- Ratray F.P. and Fox P.F. 1998. Recently identified enzymes of *Brevibacterium linens* ATCC 9174 a review. Journal of Food Biochemistry, 22:353 -373.
- Rea M.C., Görges S., Gelsomino R., Brennan N.M., Mounier J., Vancanneyt M., Scherer S., Swings J. and Cogan T.M., 2007. Stability of the biodiversity of the surface consortia of Gubbeen, a red-smear cheese. Journal of Dairy Science 90:2200-2210. DOI: doi.org/10.3168/jds.2006-377.
- Reps A. 1993. Bacterial surface-ripened cheeses. Cheese, Chemistry, Physics and Microbiology. Vol 2. 2nd Ed. P.F. Fox, Ed. Chapman and Hall, London, United Kingdom; Vol. 2, 137-172.
- Ritschard J. S., Amato L., Kumar Y., Müller B., Meile L., and Schuppler M. 2018. The role of the surface smear microbiome in the development of defective smear on surface-ripened red-smear cheese. AIMS Microbiology 4(4):622-641. DOI: doi.org/10.3934/microbiol.2018.4.622.
- Schubert K., Ludwig W., Springer N., Kroppenstedt R.M., Accolas J. P. and Fiedler F., 1996. Two coryneform bacteria isolated from the surface of french Gruyère and Beaufort cheeses are new species of the genus *Brachybacterium*: *Brachybacterium alimentarium* sp. nov. and *Brachybacterium tyrofermentans* sp. nov. International Journal of Systematic Bacteriology 46:81-87. DOI: doi.org/10.1099/00207713-46-1-81.
- Shalabi S. I. and P. F. Fox. 1987. Electrophoretic analysis of cheese: Comparison of methods. Irish Journal of Food Science and Technology 11(2):135-151.
- Seiler H. 1986. Identification of cheese-smear coryneform bacteria. Journal of Dairy Research 53:439-449.
- Singh T. K., Fox P. F., and Healy A. 1995. Water-soluble peptides in Cheddar cheese: isolation and identification of peptides in the UF retentate of water-soluble fractions. Journal of Dairy Research 62:629-640.

- Singh T. K., Fox P. F. and Healy A. 1997. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *Journal of Dairy Research* 64:433-443.
- Singh T. K., Fox P. F., Højrup P. and Healy A. 1994. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. *International Dairy Journal* 4:111-122.
- Tyanova S., Temu T., Sinitcyn P., Carlson A., Hein M., Geiger Y., Mann T. and Cox J. 2016. The Perseus computational platform for comprehensive analysis of proteomics data. *Nature Methods* 13:731-740. DOI: doi.org/10.1038/nmeth.3901
- Tyanova S., Temu T. and Cox J. 2016a. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nature Protocol* 11:2301-2319. DOI: doi.org/10.1038/nprot.2016.136
- Upadhyay V.K., McSweeney P.L.H., Magboul A.A.A. and Fox P. F. 2004. Proteolysis in cheese during ripening. *Cheese: Chemistry, Physics and Microbiology*. Edn. 3, Fox P.F., Mcsweeney P.L.H., Cogan T.M. and Guinee T.P. Elsevier London, UK; 391-433.
- Valdés-Stauber N., Scherer S. and Seiler H. 1997. Identification of yeasts and coryneform bacteria from the surface microflora of Brick cheese. *International Journal of Food Microbiology* 34:115-129. DOI: doi.org/10.1016/S0168-1605(96)01171-3.
- Wolfe B.E., Button J., Santarelli E. and Dutton M. R. J. 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* 158:422-433. DOI: doi.org/10.1016/j.cell.2014.05.041.
- Wyder M.T. and Puhán Z. 1999. Investigation of the yeast flora in smear ripened cheeses. *Milchwissenschaft* 54:330-333.
- Zarmpoutis I.V., Mcsweeney P.L.H. and Fox P.E 1997. Proteolysis in blue-veined cheeses: an intervarietal study. *Irish Journal of Agriculture Food Research* 36:219-229.
- Zhang G, Ueberheide B. M., Waldemarson S., Myung S., Molloy K., Eriksson J., Chait B.T., Neubert T.A. and Fenyö D. 2010. Protein quantitation using mass spectrometry. *Methods Molecular Biology* 673:211-222. DOI: doi.org/10.1007/978-1-60761-842-3_13.

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